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Intellectual Property Office of New Zealand
Te Pou Rāhui Hanga Hou

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 5 June 2002 with an application for Letters Patent number 519363 made by AgResearch Limited.

Dated 27 June 2003.

Neville Harris
Commissioner of Patents

519363

PATENTS FORM NO. 4

Appln Fee: $50.00

James & Wells ref: 19490/3 VM

PATENTS ACT 1953

PROVISIONAL SPECIFICATION

A NOVEL DRUG DOSING REGIMEN

We AgResearch Limited, a New Zealand company of East Street, Ruakura Campus, Hamilton, New Zealand do hereby declare this invention to be described in the following statement:

This invention relates to a novel drug dosing regimen. More particularly it relates to a novel immunisation/vaccination regimen and a delivery means therefore.

Current immunisation/vaccination regimens usually entail an initial immunisation followed by one or more booster immunisations at defined intervals. This regimen appears to be necessary for the induction of antigen-specific memory lymphocytes, particularly memory B lymphocytes.

Typical regimens for both humans and animals are, for example, immunisation at 0, 4 and 26 or 52 weeks, although immune responses vary significantly with immunisation regimens and current regimens have been designed to optimise antibody production.

In the immunisation of farm animals, a vaccination regimen of 0 and 4 weeks, for example, would involve the mustering and re-mustering of animals which is very time consuming, expensive and difficult to ensure that every animal is immunised at the right time.

Accordingly it would be desirable to have an alternative immunisation/vaccination regimen which would provide effective immunisation without the need for repeated booster immunisations. Such a drug dosing regime would also be useful for the delivery of biologically active agents other than vaccines, such as hormones, neutraceuticals, vitamins and trace-elements.

It is an object of the present invention to go some way towards achieving the above desiderata or at least to offer the public a useful choice.

According to a first aspect of the present invention there is provided a process for immunising an animal which comprises the administration to said animal of progressively increasing doses of one or more immunising agents which are released over a predetermined period of time from a delivery means which is administered to an animal on a single occasion.

Preferably said one or more immunising agents comprise an antigen/vaccine or combination of antigens/vaccines.

The antigen/vaccine may optionally comprise an adjuvant and/or a pharmaceutically acceptable carrier.

Preferably the antigen/vaccine is selected from molecules that will induce protective immunity against a disease causing organism, or functional immunity or any combination thereof.

Preferably, the progressively increasing doses comprise sequentially doubling doses of antigen/vaccine, or sequentially-increasing doses such as 25, 50, 75, 100μg.

This novel immunisation/vaccination regimen mimics the growth of bacteria during an episode of infection and the natural antibody response thereto.

The doses of the progressively increasing doses of antigen are chosen so as to illicit a favourable antibody response which includes the production of both high-affinity antibodies and antigen-specific memory lymphocytes.

Preferably, the progressively increasing doses of antigen are in the range of from 0.1μg to 1000 μg.

Preferably, the predetermined period of time at which the one or more drugs are administered is selected from hours, days, weeks or months or any combination thereof.

The delivery means preferably comprises an immunising agent delivery composition which comprises means to enable progressively increasing doses of one or more immunising agents to be released over a predetermined period of time therefrom, when said delivery means is administered to an animal on a single occasion.

The present invention further provides an unloaded delivery means comprising an unloaded immunising agent delivery composition which comprises means to enable progressively increasing doses of one or more immunising agents to be released over a predetermined period of time once said immunising agent is loaded into said immunising agent delivery composition and when said delivery means is administered to an animal on a single occasion.

The present invention further provides an immunising agent delivery composition loaded with one or more immunising agents whereby said composition comprises means to enable progressively increasing doses of said one or more immunising agents to be released over a predetermined period of time from a delivery means which is administered to an animal on a single occasion.

Preferably the immunising agents are antigens with or without adjuvant.

Preferably, the delivery composition comprises means to enable the delivery of one dose of said one or more antigens within hours/days/weeks/months of its administration and means to enable the delivery of further progressively increasing doses of the same or different antigens hours/days/weeks or months later.

In one alternative, the delivery composition comprises two or more types of microspheres or microparticles, each type of microsphere or microparticle containing a different dose of one or more antigen and comprising biodegradable material which will degrade over a known time period so that the lowest dose of antigen is released from a first type of microsphere or microparticle at a set time after administration, followed by the next highest dose of antigen at the next predetermined time etc.

In another alternative, the composition may be located within known or novel drug delivery devices, for example, the drug delivery device may be of a multi-compartmental capsule type containing progressively increasing doses of one or more immunising agents within the compartments, said device comprising an outer wall made of a biodegradable substance which degrades over a pre-set period of time to release the smallest dose of drug(s), and one or more inner compartmental walls made of the same or different material that degrade over a longer period of time to release progressively increasing pulses of the bioactive ingredient.

The biodegradable material of the outer wall may be selected from the group comprising cholesterol/lecithin, polylactide and/or polyglycolide copolymers, one or more of a number of cellulose polymers, polyacrylic acid, polymethylmethacrylate, cross-linked polyacrylic acid, polycaprolactone, polyvinylpyrrolidine, polyvinylalcohol, polyethylene glycol, agarose, DEAE dextran microspheres, starch microspheres and/or albumin microspheres

The biodegradable material of the inner compartmental walls may be selected from the above named compounds.

The pre-set period of time within which the outerwall and inner compartmental walls degrade may be selected from hours, days, weeks or months.

Alternatively, the drug delivery device may comprise a capsule having an osmotic pump therein as is known from ALZET Technical Information Services, ALZA Corporation, 950 Page Mill Road, P.O.Box 10950, Palo Alto, CA 94303-0802, USA.

Other known drug delivery devices may also be used as vehicles to deliver the novel drug dosing regimen of the present invention.

Alternatively, the drug delivery device may comprise a bioerodable device that releases progressively increasing amounts of antigens as it erodes.

The progressively increasing doses of the one or more immunising agents may be administered to an animal by way of injection, ingestion or implantation.

The administration, ie injection, ingestion or implantation, of the immunising agents according to the novel dose regimen of the present invention preferably takes place shortly after the birth of an animal, or when maternally-derived antibody has decreased sufficiently for the young animal to be able to respond to the vaccination, and provides immunity without the need for further booster administration.

The present invention further provides a drug delivery device comprising an immunising agent delivery composition according to the invention.

The drug delivery device is selected from the group comprising, multi-compartmental capsules and capsules having as osmotic pump therein or any other known drug delivery device to which the novel drug delivery regimen is incorporated.

Alternatively, the present invention provides a process for administering to an animal progressively increasing doses of one or more biologically active agents which are released over a predetermined period of time from a delivery means which is administered to an animal on a single occasion.

Preferably said one or more biologically active agent comprise drugs, such as antibiotics, anthelmintics, peptides, proteins, carbohydrates, DNA, RNA, hormones, neutraceuticals, vitamins or trace-elements.

The biologically active agents may optionally comprise a pharmaceutically acceptable carrier.

Preferably the progressively increasing doses comprise sequentially doubling doses of biologically active agent, or sequentially increasing doses such as 25, 50, 75, 100 or 4, 8, 32, 150 active units. The doses are chosen so as to elicit a desired response and are administered over a predetermined period of time from hours, days, weeks and months or any combination thereof.

The delivery means comprises a biologically active agent delivery composition which comprises means to enable progressively increasing doses of one or more biologically active agents to be released over a predetermined period of time therefrom, when said delivery means is administered to an animal on a single occasion.

The delivery composition is as is described above for the immunising agent delivery composition except that the composition is either loaded or unloaded with one or more biologically active agent other than an immunising agent.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

DEFINITIONS

Eg 95; A vaccine molecule which protects sheep against acquisition of the cysts of Echinococcus granulosus (hydatid Disease).
GST Eg 95; The fusion partner of the recombinant fusion protein is glutathione-S-transferase (GST) from Schistosoma japonicum.
PIRS; Progressively Increasing Release System.
Quil A: A purified extract from the bark of a South American tree (Quillaia saponaria) which is used as an adjuvant for the vaccine Eg 95.

The present invention will be further described with reference to the figures of the accompanying drawings in which:

Figure 1 shows the antibody response to conventional immunisation regimen in sheep having 50μg of Eg95 vaccine administered at 2 months and 3 months of age and a booster at 8 months of age, compared to control sheep who received no vaccine.

Figure 2 shows the antibody response over time to a single injection of 50 or 100μg of Eg95 vaccine in control sheep compared to an injection of an osmotic pump drug delivery device containing a single dose of 50 or 100μg of Eg95 vaccine.

Figure 3 shows individual data for four sheep injected daily with progressively increasing doses of Eg95 recombinant antigen and Quil A adjuvant (PIRS) in comparison to four sheep receiving two injections one month apart of 50μg Eg95 and Quil A (control).

Figure 4 shows the combined data of the sheep of figure 3 with nineteen antibody measurements taken over 18 months.

Figure 5 shows the antibody response of sheep to progressively increasing doses of GST Eg95 vaccine, 25μg, 50μg, 75μg and 100μg administered at weekly intervals (PIRS) compared to two control groups, "control +" which were given two doses of 125μg GST Eg95 four weeks apart and "control -" which were given Quil A adjuvant only in two doses at four weeks apart.

Figure 6 shows the antibody response of sheep to progressively increasing doses of GST Eg 95 vaccine, 4μg, 8μg, 32μg and 150μg administered at weekly intervals with a standard amount of Quil A (Group 1) or with increasing amounts of Quil A (Group 2) compared to two control groups given two doses of 50μg GST Eg 95 being either urea depleted (Group 3) or non-urea depleted (Group 5) and standard amounts of Quil A four weeks apart and a negative control (Group 4) which had no injections.

EXPERIMENTAL DETAILS

1. Conventional Vaccination Regimen

Two injections were given, one month apart, subcutaneously in the neck region. The vaccine consisted of 50μg of Eg95/GST fusion protein active ingredient and 1mg of Quil A (Superfos Biosector, Denmark), which was reconstituted from lyophilised batches of 50 doses. A new batch was reconstituted each time. Vaccination was repeated 6 months after the second vaccination, to promote an anamnestic response. The vaccine (China 5000) was formulated on 12/11/96 and made from inclusion bodies grown on 1/9/96. The formulation for the China 5000 Vaccine used in the trials is as follows:

Total Protein = 5.4mg/mL

%Eg95 protein = 22.3%

Total Eg95 protein = 5.4 x 0.223 = 1.2mg/mL

50 doses were made up in 10mL at 50μg/mL = 2500μg Eg95

= 2.08mL of solubilised inclusion bodies + 7.92mL formulation buffer + 50mg of QuilA

Formulation Buffer **PBS pH 6.8**

$Na_2HPO_4.12H_2O$	4.656 g/L
$NaH_2PO_4.2H_2O$	1.092 g/L
NaCl	8.5 g/L
Phenonip (NIPA Laboratories Ltd)	2.5 mL/L
Water for Manufacturing to 1 litre	

Sheep were bled regularly from the jugular vein, using "vacutainer" equipment, and sera was stored at -20 C until required for testing. Absorbances of the ELISA test are shown in Fig. 1. The antigen used in the ELISA was Eg95/HIS. The polyhistidine fusion protein (Quiagen) did not react with antibody generated against the GST portion of the vaccinating molecule.

RESULTS

The conventional protocol for delivering vaccine is two injections, given approximately one month apart. Ideally, the antibody response to the first injection has begun to wane before the second injection - the second injection is then more successful in stimulating clonal expansion of antibody-forming cells. A third injection given 3-12 months later causes the so-called anamnestic response, where increased clonal expansion occurs and long-lived antibody-forming cells are stimulated. Figure 1 demonstrates this principle.

2. Osmotic Pump Trial

The aim of this trial was to determine what level of continuous release of antigen and adjuvant was necessary to maintain a level of antibody in serum that was equivalent to that stimulated by conventional vaccination technology. Based on this information, a slow-release delivery device would be devised to perform the same function as the osmotic pumps.

ALZET osmotic pumps were chosen that delivered 2.5µl/hour for 28 days, and after filling with a suitable dosage of antigen, they were surgically-implanted

subcutaneously in the shoulder region. After 28 days, a new pump was inserted into an area close to the original pump, and the original was withdrawn.

Four 6-month-old lambs were vaccinated conventionally with either 50 or 100μg of Eg95/GST with Quil A as shown in Table 1, and another 4 were implanted with pumps that delivered the same amount of antigen as above, but continuously released over 28 days. At the end of the 28 days, the conventional vaccination was repeated , and the pumps were replaced. After a further 28 days, the second series of pumps were withdrawn. Blood was collected and serum stored for ELISA analysis, at fortnightly intervals, and the at 2 and 3 months after the end of the vaccination period.

TABLE 1

Group	Sheep	Vacc.	Route	Quil A	Eg95	PBS	Volume
1	4966	inject	s/c	1000μg	50μg	-	0.2ml
1	4970	inject	s/c	1000μg	50μg	-	0.2ml
2	4965	inject	s/c	2000μg	100μg	-	0.4ml
2	4985	inject	s/c	2000μg	100μg	-	0.4ml
3	4980	pump	s/c	1000μg	50μg	2.1ml	2.3ml
3	4971	pump	s/c	1000μg	50μg	2.1ml	2.3ml
4	4968	pump	s/c	2000μg	100μg	1.9ml	2.3ml
4	4986	pump	s/c	2000μg	100μg	1.9ml	2.3ml

RESULTS

Figure 2 demonstrated that continuous release of antigen and adjuvant from an osmotic pump can cause antibodies to rise to levels above that achieved with conventional vaccination. However, the cessation of antigen release resulted in a return to zero levels of antibody production. The animals receiving a continuous priming dose of antigen are fully-primed for a secondary response (data not shown). However, the aim of these experiments was to devise single-shot antigen presentation.

3. Progressively increasing release system for vaccines (PIRS I and PIRS II)

There is no record of research that attempts to duplicate a proliferative disease, which tends to provide antigen exponentially to the host until after the host has fully-responded and destroyed the pathogen. The aim of a regimen of progressively increasing release of antigen was investigated as a means of achieving the high titres

normally resulting from the conventional 2 injections at, for example, 0 and 1 month of vaccination. A successful demonstration using carefully-graded daily injections will allow for the duplication of this style of antigen release in a biodegradable pellet of novel constructions.

Protocol:

Arithmetically increasing vaccination.

Preparation of the vaccine. (SC1300, His antigen solubilised in sarkosyl)

The antigen for the vaccine was obtained from fermentation batch, 11/4/96. Inclusion bodies isolated from the culture were solubilised in 1.5% Sarkosyl. The original concentration of the sarkosyl preparation had been approximately determined from page gel analysis to be 7.9 mg/ml. To obtain a 50μg/ml vaccine the sarkosyl preparation needed to be diluted by a factor of 158. It was decided that a 0.5ml dose would be given and therefore the vaccine concentration was made up to 100μg/ml in formulation buffer. PBS, pH6.8: $Na_2HPO_4.12H_2O$ (4.656 g/L); $Na_2H_2PO_4.2H_2O$ (1.092 g/L); NaCl (8.5 g/L); phenonip (2.5 mL/L); in d.H_2O.

Dilution of Sarkosyl preparation:

254 μl of the sarkosyl solubilised inclusion bodies, was added to 20mls of formulation buffer.

Quil A was also added to the vaccine at a concentration of 2 mg /ml .

From the 100μg/ml vaccine 2.5 mls was removed and added to another 2.5 mls of formulation buffer. This was to prepare a vaccine with antigen concentration of 50μg/ml. This double dilution procedure was repeated until a vaccine was prepared with antigen concentration of 0.000019 μg/ml.

Vaccination Procedure:

4 sheep were injected daily with arithmetically-increasing (double dilution) doses of Eg95 recombinant antigen and Quil A as adjuvant (PIRS) for 20 days as follows:

On Day 1 0.000095µg of antigen, finishing on Day 20 with 50µg of antigen and 1mg Quil A (see Table 2 below).

(The Quil A was doubly diluted also, by making up 200µg of antigen and 4mg of Quil A, and doubly diluting this mix 19 times). Four control sheep received 50µg Eg95 and

1 ml Quil A on Days 1 and 30. Serum was taken from all sheep weekly to monitor antibody responses. Responses were tested against Eg95 using the ELISA test. Monthly serum samples were taken and monitored for the following 18 months.

TABLE 2

Day No.	Dose (μg)
1	0.000095
2	0.00019
3	0.00038
4	0.00076
5	0.0015
6	0.003
7	0.006
8	0.012
9	0.024
10	0.048
11	0.096
12	0.192
13	0.38
14	0.78
15	1.56
16	3.12
17	6.24
18	12.5
19	25.0
20	50

RESULTS

Figures 3 and 4 compare the results of conventional vaccination with a progressively increasing daily exposure of antigen to sheep. Sheep receiving conventional vaccination were numbered 4701, 4679, 4699 and 4697. Sheep receiving the simulation of progressively-increasing release of antigen (PIRS I and II) were numbered 4696, 4675, 4677, 4681. Elisa results showed antibody titres had dropped in the PIRS vaccinated sheep.

It was decided to boost all the sheep, both controls and PIRS sheep, with a single vaccination of 50λg Eg95 and 1000λg Quil A.

The vaccine used was formulated on the 10/11/95 as part of a batch of vaccine. The striking similarity of the antibody responses between the two groups indicates that PIRS is likely to be as effective as conventional vaccination in stimulating a prolonged response and an anamnestic response. Possibly one more injection should have been added to the PIRS to raise the antibody levels to those of the conventional vaccination, especially as following a booster injection 6 months later, it appears that the height of the initial antibody absorbance may have a significant influence on the height of any subsequent anamnestic response.

4. Progressively increasing release system for vaccines (PIRS III)

This trial was carried out to simulate the effect of pulse releases of increasing doses of the vaccine GST Eg95 on the antibodies titre of sheep.

The vaccine used for the trial was formulated on the 12/11/96, and was made from the fermentation batch grown on the 17/9/96. The vaccine was formulated as described above.

Details of all vaccines and new formulations are described in "*Echinococcus granulosus* Vaccine Production Books available at AgResearch Wallaceville upon request.

Protocol:

The trial was designed as follows:

Group 1 (5 sheep) received 25μg, 50μg, 75μg and 100μg of GST Eg95 at weekly intervals (PIRS).
Group 2 (5 sheep) received 2 doses of 125μg GST Eg95 four weeks apart (control +).
Group 3 (5 sheep) received 2 doses of 1mg Quil A, four weeks apart (control -).

RESULTS

PIRS III was designed to lend itself to effective manufacture. As mentioned above, the progressively-increasing doses were given on 4 occasions, each one week apart. Figure 5 shows that the PIRS III was clearly more effective in stimulating an early antibody response compared to conventional vaccination, and that the eventual

outcome of both vaccination regimes were similar. There is clearly an advantage for disease control if an early and rapid response to the vaccine can be stimulated.

5. Progressively increasing release system for vaccines (PIRS IV)

The trial was carried out to establish whether a conventional protocol of two injections could be improved by a progressively increasing release system (PIRS) protocol of four injections.

The vaccine (GST Eg 95) used for Groups 1-3 of the trial was Antigen #HYD/024 which comprised:

Total protein = 4.1mg/mL

Eg95 protein = 25% of total protein by densitometry = 1mg/mL

Using the stirred cell and a PM10 membrane the antigen was concentrated and re-diluted 4 x using glycine buffer to remove the urea.

1mL of HYD/024 contains 1mg Eg95 ∴ 5mL of HYD/024 contains 5mg Eg95 = 100 doses at 50µg/dose. The antigen was aliquoted in 5mL amounts and freeze-dried without Quil A. This urea-depleted antigen was used for groups 1,2 and 3.

The vaccines were made up into 15ml aliquots and frozen, except for Day 0.

The vaccine for group 5 was made from HYD/024 starting material but was non-urea deplete.

Protocol:

The trial was designed as follows:

Group 1 (10 sheep) received 4µg, 8µg, 32µg and 150µg of urea depleted GST Eg 95 and 0.5mg of Quil A at weekly intervals.

Group 2 (10 sheep) received 4µg, 8µg, 32µg and 150µg of urea depleted GST Eg 95 and 0.04mg, 0.08mg, 0.32mg and 1.50mg Quil A at weekly intervals.

Group 3 (10 sheep) received 2 doses of 50µg urea depleted GST Eg 95 and 1mg Quil A four weeks apart.

Group 4 (10 sheep) received no injections (negative control).

Group 5 (10 sheet) received 2 doses of 50μg non-urea depleted GST Eg 95 and 1mg Quil A four weeks apart.

All sheep were artificially challenged with live E. granulosus eggs, three months after the second vaccination of Groups 3 and 5, and the subsequent necropsy examined to compare the levels of protection. The level of immunity was also determined from the antibody levels which were measured from blood samples taken from all groups from Day 0 and weeks 1, 2, 3, 4, 6 and 8.

RESULTS

Figure 6 shows that the PIRS IV (Groups 1 and 2) was clearly more effective in stimulating an early antibody response at four weeks compared to conventional vaccination (Groups 3 and 5) where the maximum response was not observed until six weeks. Further, the trial shows that the eventual outcome of both vaccination regime were similar. Results of the necropsy examination will be available to check the level of immunity provided by the vaccination regimens at the cellular level.

CONCLUSION

The three PIRS systems (I and II; III and IV) look to be effective in stimulating an effective primary/secondary antibody response which is equal to the widely-separated primary and secondary responses of conventional vaccination.

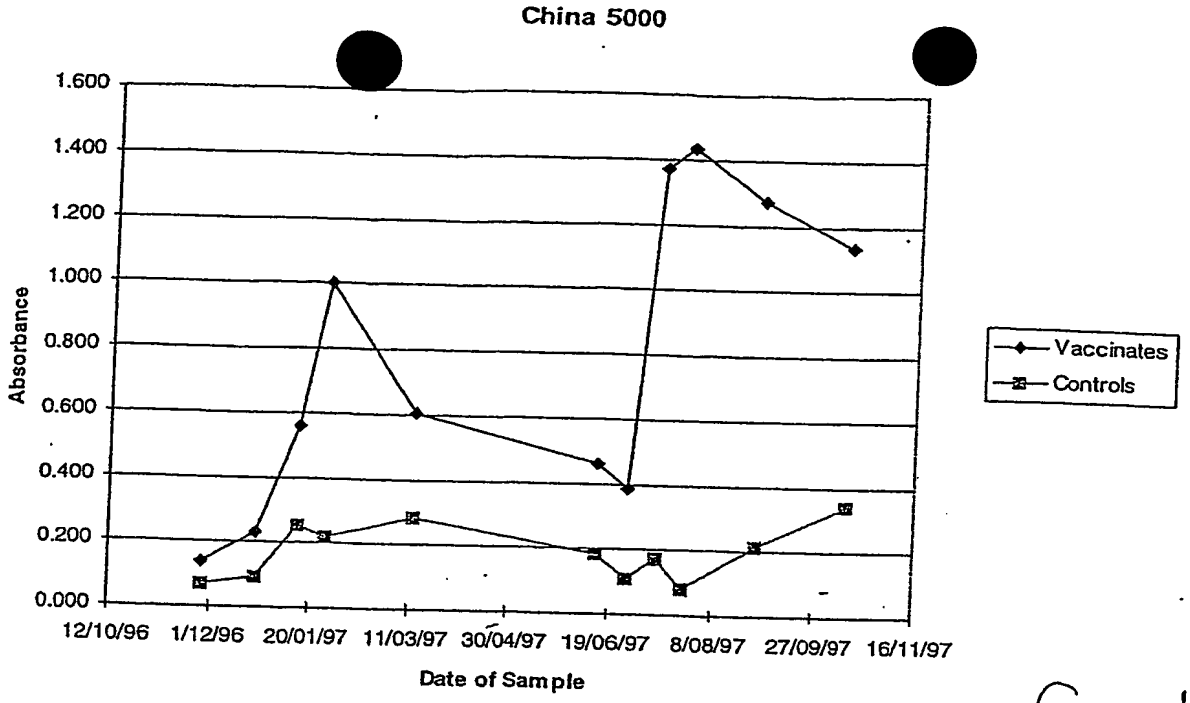
The daily doubling of antigen exposure system lends itself to simulation using a progressively-increasingly bioerodable matrix. The four progressively-increasing releases of antigen lend themselves to an erodable matrix with layers of antigen/adjuvant incorporated.

It will be appreciated that it is not intended to limit the invention to the above examples only, many variations, such as might readily occur to a person skilled in the art being possible without departing from the scope of the invention.

AGRESEARCH LIMITED

By their Attorneys

JAMES & WELLS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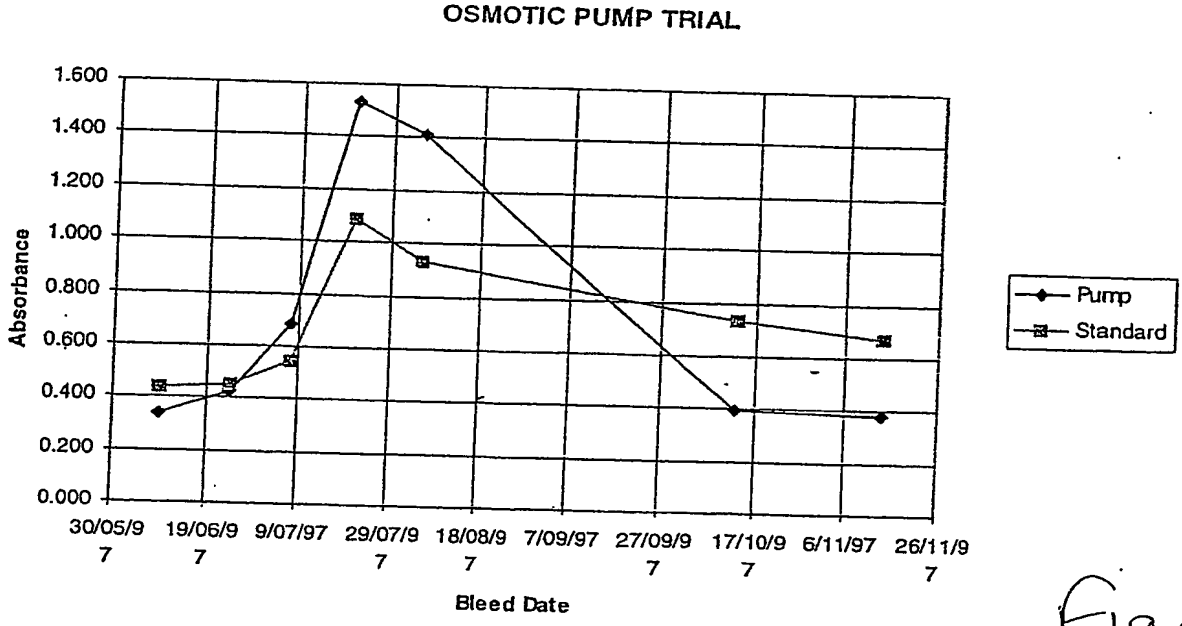
Vaccinates
Controls

Fig 1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18/08/97
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Bleed Date
Pump
Standard

Fig 2

PIRS I Trial

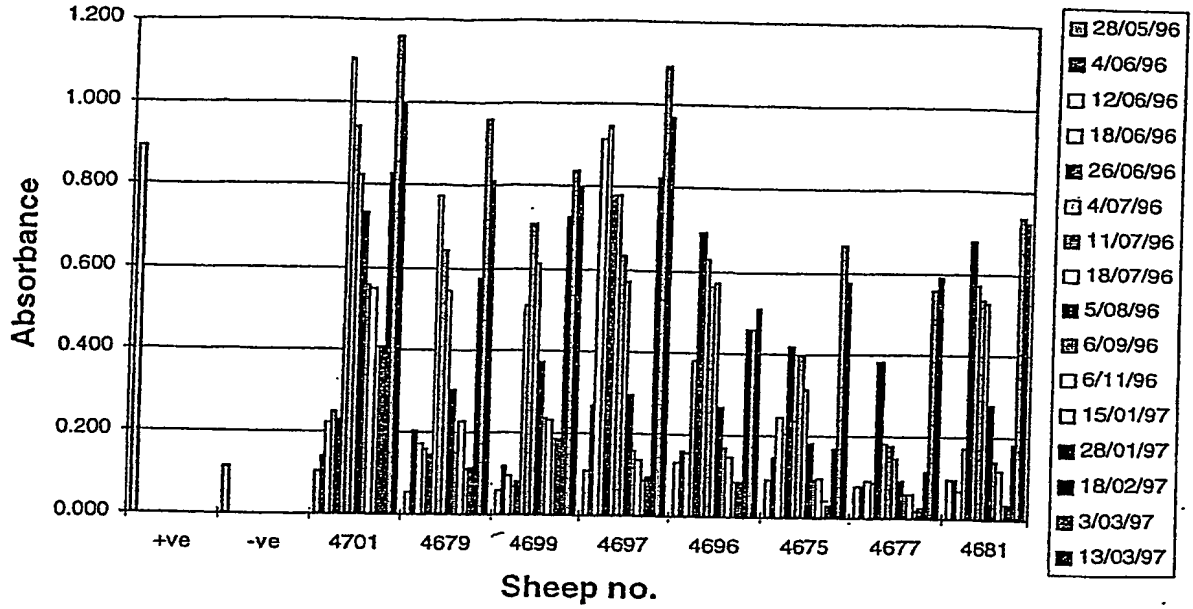

Fig 3

Controls vs PIRS II

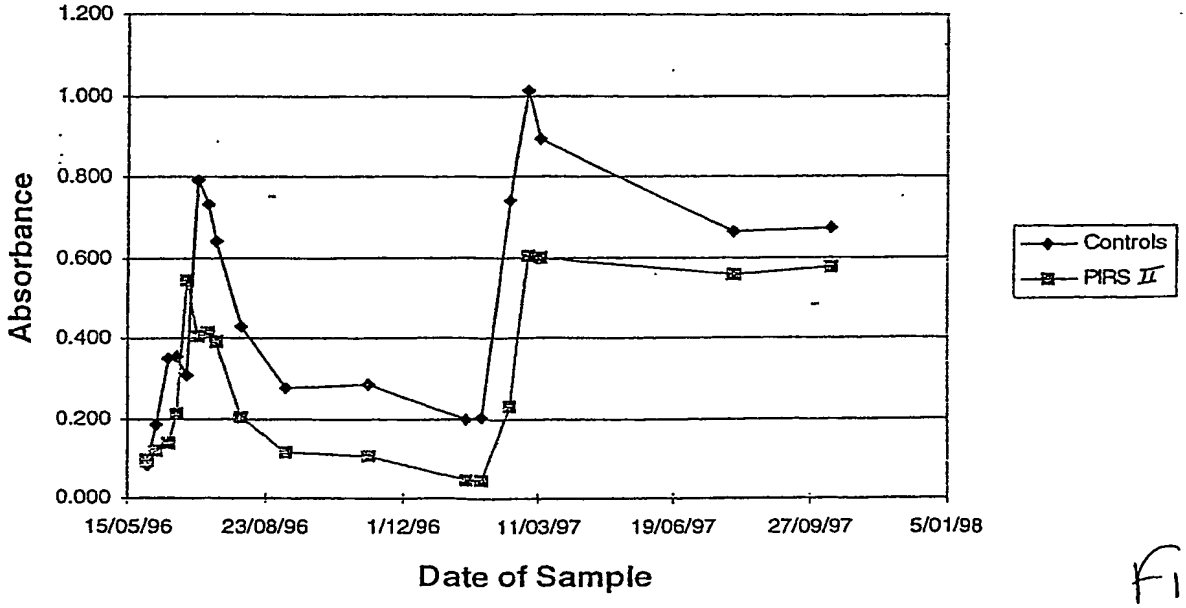

Fig 4

PIRS III ELISA ON POOLED SERA

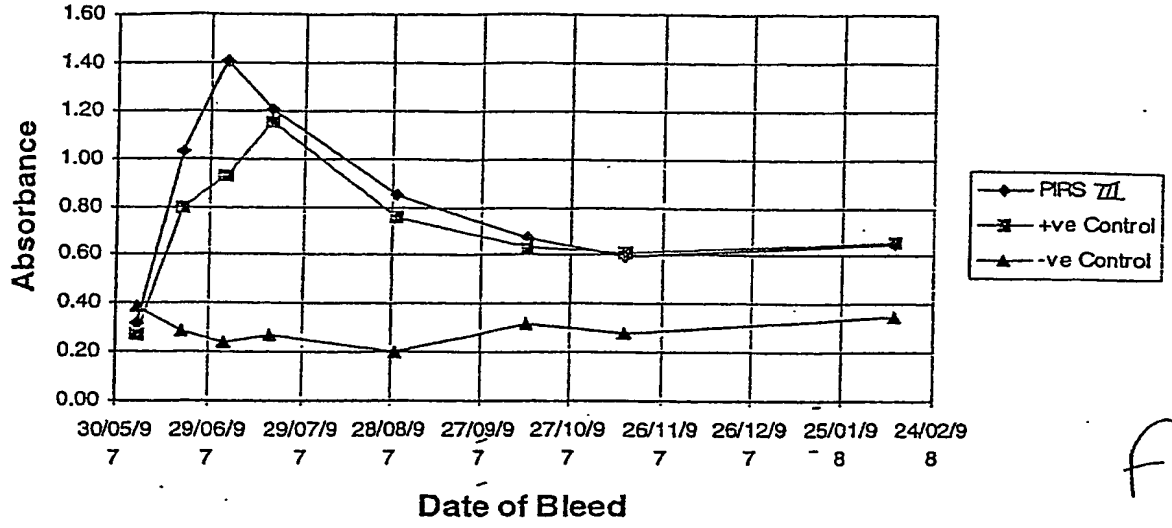

Fig 5

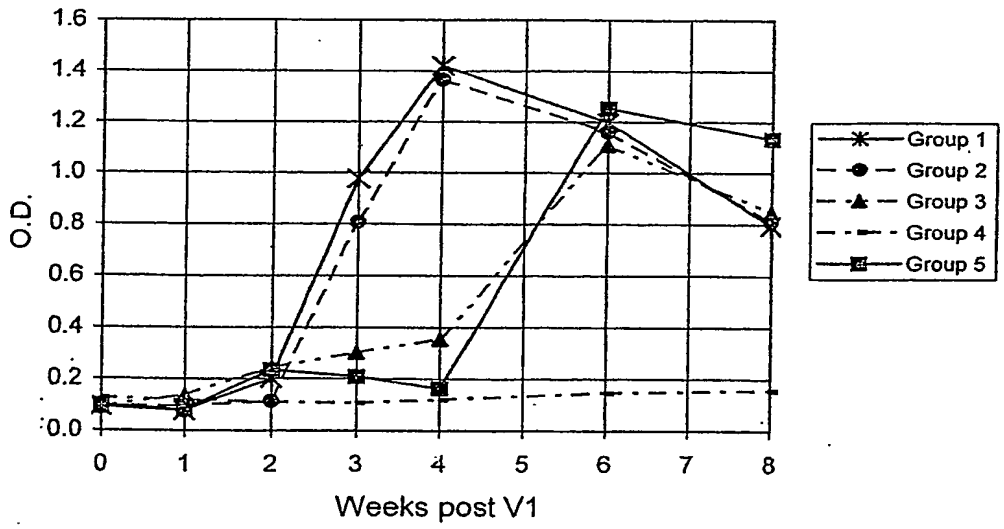

Fig 6